AD_____

Award Number: W81XWH-10-1-0257

TITLE: Commensal Gut-Derived Anaerobes as Novel Therapy for Inflammatory Autoimmune Diseases

PRINCIPAL INVESTIGATOR: Veena Taneja

CONTRACTING ORGANIZATION: Mayo Clinic
Rochester, MN 55905

REPORT DATE: May 2012

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
01-05-2012	Annual	15 APR 2011 - 14 APR 2012

4. TITLE AND SUBTITLE
Commensal Gut-Derived Anaerobes as Novel Therapy for Inflammatory Autoimmune Diseases

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-10-1-0257

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Veena Taneja

E-Mail: taneja.veena@mayo.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Mayo Clinic
Rochester, MN 55905

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Predisposition to rheumatoid arthritis (RA) is associated with the presence of genetic factors, HLA class II molecules, DR4 and DQ8, being the strongest. Patients with RA show an imbalance of gut microbiota suggesting its role in regulation of disease. We have used HLA-DR4/ DQ8 mice to test our hypothesis that treatment with commensal bacteria like Prevotella histicola can modulate Collagen-induced arthritis (CIA). In vitro study showed that treatment of mice with P. histicola in CII-immunized mice led to suppression of antigen-specific immune response and reduction in production of inflammatory cytokines. We carried out rtPCR for various cytokines and regulatory proteins in guts of mice treated with p histicola and controls. Our data showed that treatment with p histicola led to an increase in Tregulatory protein FoxP3 as well cytokine IL-10, a cytokine produced by Treg cells and suppression of pro-inflammatory IL-23. To determine if susceptibility is associated with proinflammatory cytokines in naïve mice, we analyzed cytokines in jejenum of arthritis-susceptible *0401 and –resistant *0402 mice. Our data showed that arthritis-susceptible mice had higher level of proinflammatory cytokines compared to resistant mice. These data suggest that P histicola induced immune responses in the gut can induce tolerance in periphery leading to systemic immune suppression and protection from arthritis and may be a potential target for therapy.

15. SUBJECT TERMS
No subject terms provided.

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRMC
U	U	U	UU	12	**19b. TELEPHONE NUMBER** *(include area code)*

Table of Contents

Page

Progress Report
This progress report is from Feb, 2011 to April 30, 2012.

Introduction
Rheumatoid arthritis is associated with the presence of certain MHC alleles, HLA-DR4 and DQ8 offering the strongest association (1, 2). Analysis of fecal microbiota of patients with RA showed significantly less *Bifidobacteria* and bacteria of the *Bacteroides-Porphyromonas-Prevotella* group, *B. fragilis* subgroup, and the *E. rectale – C. coccoides* group than the fecal microbiota of patients with non-inflammatory Fibromylgia (3). Since these bacterial species are known to belong to the most common genera and groups in the human fecal microbiota, their absence in RA patients might suggest a protective role of these commensal bacteria in RA Using mice expressing HLA-DR4 and DQ8, we generated a model of collagen-induced arthritis (CIA) that mimics human rheumatoid arthritis in autoantibody profile and sex-bias (4-6). In this proposal we aim to investigate immunomodulatory properties of *P histicola* to suppress inflammatory autoimmune diseases in HLA-class II Tg mice. The major goal of these experiments is to determine if mucosal immune tolerance is a viable option for the treatment of rheumatoid arthritis.

Our preliminary data showed that HLA-DQ8 mice immunized with type II collagen and fed with *P. histicola* develop much lower incidence of arthritis suggesting immunosuppressive properties of P histicola. Since oral treatment with *P histicola* led to suppression of systemic immune response, it shows that interaction of commensal bacteria at mucosal surfaces can modulate immune response in periphery.

In the second year we show that P. histicola can protect DQ8 and DR4/DQ8 mice from arthritis in protective and therapeutic protocol. On the other hand, treatment with a control bacteria Prevotella melanogenics did not result in protection from arthritis. We further show that arthritis is regulated via generation of T regulatory cells and regulatory DCs. The treatment led to increased production of regulatory cytokines like IL-10 with lower levels of IL-17. Further, rtPCR for various cytokines and chemokines showed that treatment led to lower expression of pro-inflammatory IL-23. To determine if gut immune system is different in naïve mice we used DRB1*0401 mice that show sex-biased arthritis and also *0402 mice that are resistant to arthritis (6). Our data shows that gut immune system is different between male and female *0401 mice and also between *0401 and *0402 mice suggesting a crucial role of gut in pathogenesis (7, 8). This also confirms our hypothesis that arthritis can be modulated via gut and such a therapy may be possible in humans.

Progress Report (SOW)

Milestone 1-7 were achieved in last year progress report.

SOW#1-i) Sections of paraffin blocks and frozen blocks from CIA (arthritis) group and staining with hematoxylin and eosin. ELISA for antibodies

Mice were immunized with type II collagen (CII) to induce arthritis and the fed them P histicola on alternate days 2 weeks following immunization. Next we tested mice in preventive protocol.

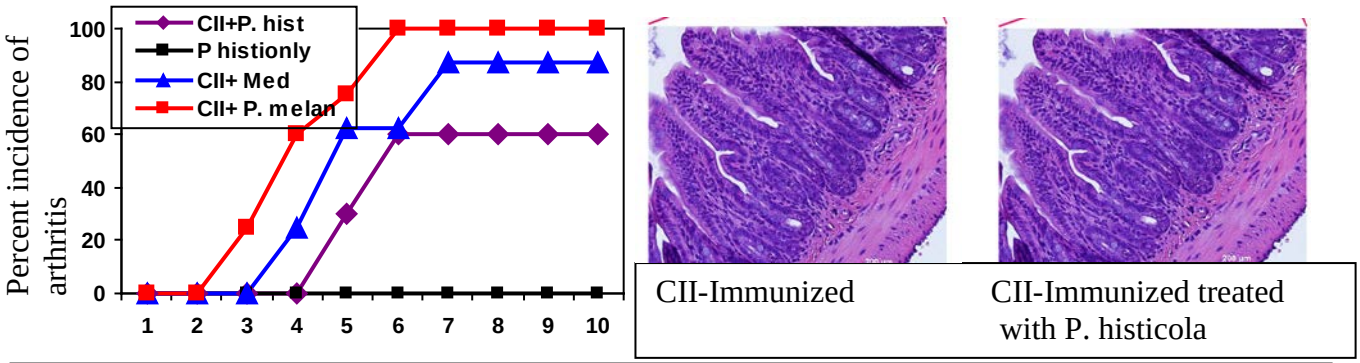

Fig1 Treatment with P. histicola of mice induced for collagen-induced arthritis suppresses disease incidence while a control bacteria P. melanogenica did not show suppressive activity. P. histicola alone did not lead to arthritis. CII-immunized and treated mice did not show any gut pathology.

Mice were fed P histicola 12 days prior to immunization with type II collagen. Mice immunized with type II collagen and fed media without bacteria as well as mice fed bacteria without CII immunization were used as controls. Disease phenotype in CIA model is characterized by paw swelling, scored on scale of 0-3. Our data showed that both preventive and therapeutic protocol suppressed disease incidence and antibodies to Collagen II. We used a control bacteria P. melanogenica; it did not show any suppressive activity. To ensure that treatment of mice did not result in any pathology of the gut, we did histopathology. P. histicola did not cause pathology in the gut (Fig1).

1-j) compilation of in vivo data and T cell response and cytokine data in CIA (arthritis) model (20th month)

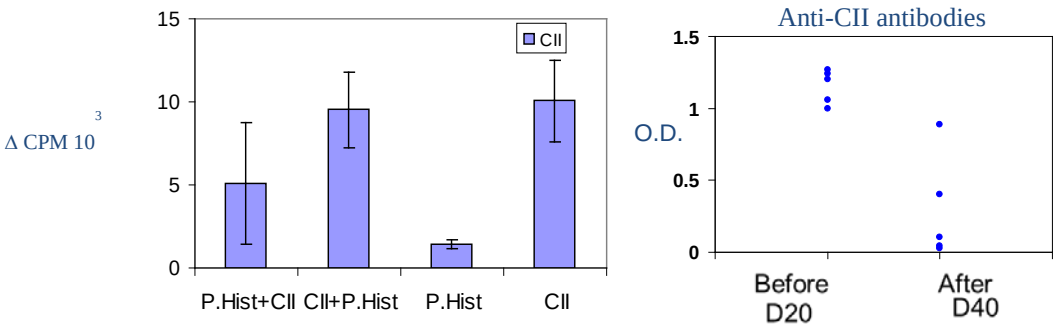

Fig 2 DQ8 mice were immunized with type II collagen (CII) and treated with P. histicola showed suppression of T cell response when treatment was before immunization. Autoantibodies to CII were significantly reduced in P. histicola treated mice. Sera collected at day 20 after immunization with CII, and day 40 after treatment started at day 21 were tested by ELISA.

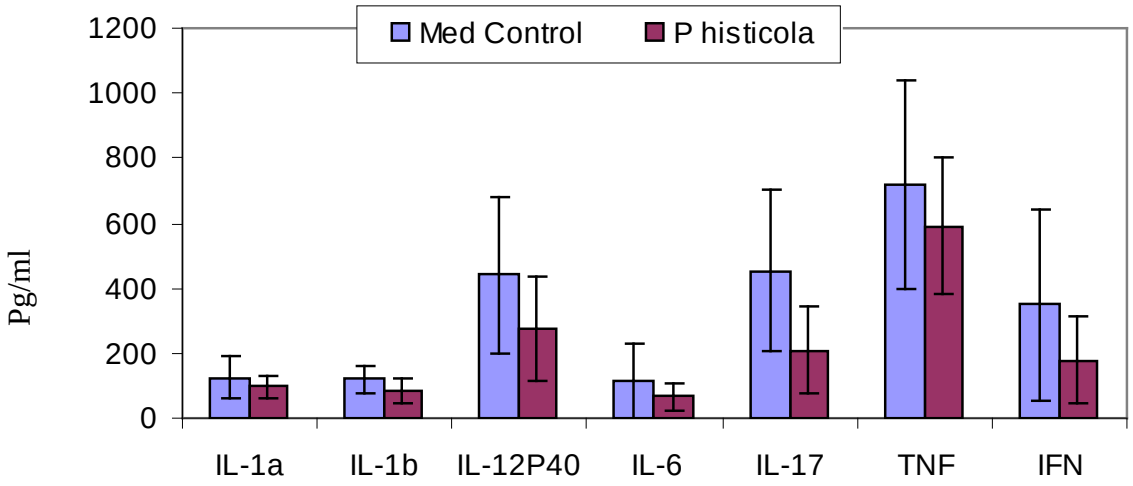

Fig 3 Cytokines in medium treated and P. histicola treated mice showed suppression of proinflammatory cytokines.

In vivo data is compiled in Fig 1. T cell response was significantly decreased when mice were first treated and then immunized with CII compared to untreated mice. However, treatment after CII immunization did not significantly reduce T cell response suggesting regulation of disease could be via other regulatory mechanism.

Aim#2 Mechanism of anti-inflammatory action of commensal bacteria *P histicola.*
2a, 2c, 2d) Heat killed bacteria and its immunomodulatory effect
Experiments with EAE model did not show any difference in heat killed and live bacteria. Considering this, this experiment was not carried out for CIA studies as while EAE model can be done in one month, CIA takes 4-6 month to complete. It was decided not to waste valuable mice and manpower.

2b, 2f, 2g) Characterization of Tregulatory cels from intestine and periphery and cytokines.

***Prevotella histicola* modulates antigen-specific responses via generation of T regulatory and**

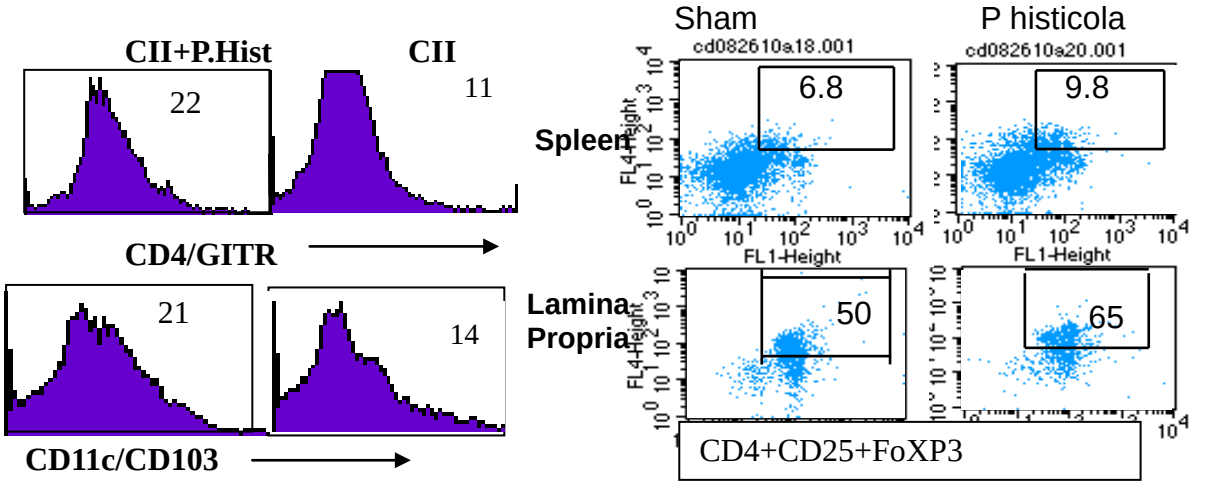

Fig 4 Treatment of DQ8 mice with P.histicola resulted in generation of regulatory DCs and T regulatory cells as observed in splenic and lamina propria isolated cells. Various cells were enumerated by FACS analysis.

regulatory DCs. : Since collagen specific T-cell responses play an important role in disease pathogenesis of CIA, we investigated the effect of treatment on T cell proliferation. Treatment led to suppression of T cell response and a much lower production of proinflammatory Th1 (IL-1, TNF and IFN) as well as Th17 (IL-12(p40), IL-17, IL-6) cytokines compared to mice immunized with CII and fed media without bacteria. These studies suggested *P. histicola* may be able to generate systemic suppression via mucosal immune regulation. We tested various cells in CII-immunized and mice immunized and treated with bacteria in spleen and lamina propria. As shown in Fig4, both spleen and lamina propria showed an increase in T regulatory, CD4+CD25+FoXP3 and CD4+GITR+, cells as well as suppressive DCs that express CD103. These studies suggest that treatment with P histicola leads to generation of regulatory DCs that activates more T regulatory cells, these T cells and DCs can produce regulatory and anti-inflammatory cytokines. Both T reg and reg DCs can migrate into the systemic immune system.

***Prevotella histicola* regulates systemic immune response via DCs and production of IL-10.**

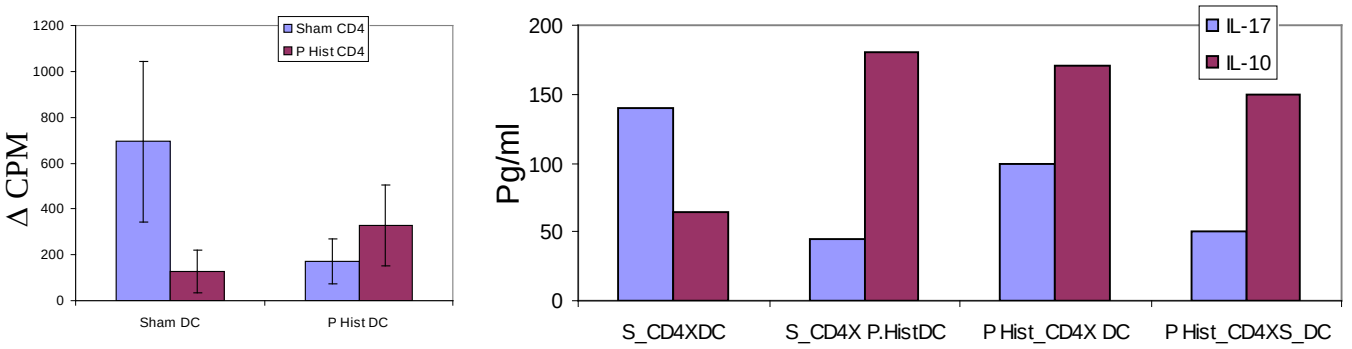

Fig5 P. histicola suppresses systemic immune response via dendritic cells. Mice treated with P. histicola or medium (Sham) were immunized with CII. Dendritic cells (DCs) and CD4 T cells were isolated from spleen and in vitro challenged with CII in a criss-cross culture. As shown in right panel, DCs from treated mice led to suppression of CD4+ T cell response in vitro. Also, when DCs and CD4+ cells of treated mice were cultured, there was increased production of IL-10 (right panel).

Mice treated with *P. histicola* and immunized with CII had DCs that when cultured with CD4 cells from treated or Sham mice suppressed immune response when challenged in vitro with CII. Supernatants from these cultures showed that treated DCs and CD4 led to production of IL-10 while IL-17, proinflammatory cytokine, was suppressed in comparison to sham treated mice that produced more IL-17 and lower IL-10. These studies suggest that *P. histicola* treatment has led to generation of regulatory DCs phenotypically and functionally (Fig 4,5).

SOW 2h, 2i Primers and rtPCR

Next we determined the effect of treatment in jejenum of DQ8 mice and compared to control by doing rtPCR for various cytokines. In addition, we also used naïve *0401 and *0402 mice to determine if gut immune system can impact susceptibility in association with genotype.

rtPCR for cytokines in jejenum: We did rtPCR for various cytokines in jejenum of mice treated and immunized with CII and those only immunized with CII using published primers. As shown in Fig 6, mice treated with *P.histicola* showed a much higher expression of anti-inflammatory cytokines, IL-4 and IL-10 and lower expression pro-inflammatory cytokine IL-23 further confirming that treatment modulates arthritis via gut As shown above, *P.histicola* alone did not cause arthritis or any pathology suggesting, this might be a good treatment for clinical trials in RA patients.

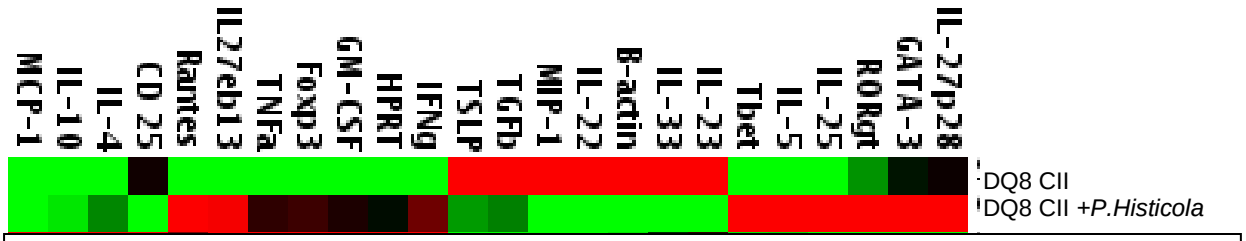

Fig 6 rtPCR for cytokines in jejenum of DQ8 mice immunized with CII and treated with P. histicola compared to CII immunized mice showed differences in pro and anti-inflammatory cytokines.

***0401 female mice show different jejenum profile than males.**

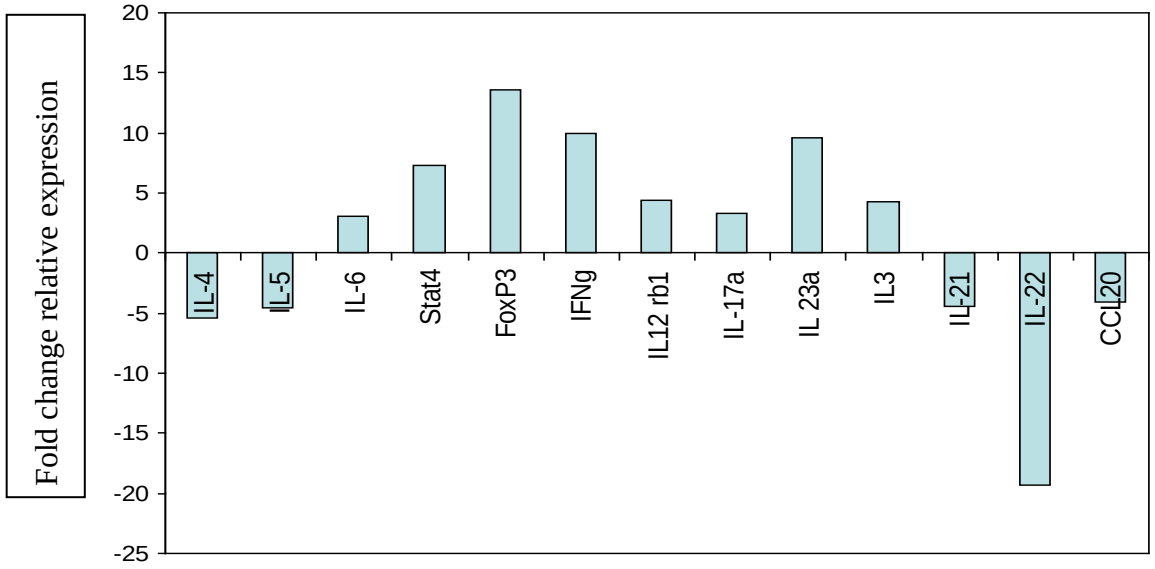

Fig 7 Comparison of fold change in gene transcript levels between *0401 females and males.

We tested the jejuna of naïve mice for expression of cytokine and chemokine transcripts involved in the Th17 regulatory network by rtPCR (Figure7). Susceptible *0401 females showed a distinct cytokine and chemokine profile as compared to males that was characterized with a significant increase in IL-23α and IFNγ along with a decrease in the regulatory cytokines IL-4, IL-22 and CCL20. Similarly, *0401 females showed more than 3 fold increased gene transcripts for Th17 cytokines IL-17, IL-23, IL-6 and Th1 cytokines IFNγ, Stat 4 and TBX21 while *0402 females had

several fold increase in genes regulating Th2 cytokines and regulatory networks like ICOS, GATA3 and IL-4. *0401 male mice did not show an increase in transcripts for TH17 encoding genes compared to *0402 mice.

Our data showed a bias towards TH1/TH17 cytokine expression with significant decrease in cytokine gene transcripts required for negative regulation of Th17 profile, like IL-4, IL-21 and IL-22, in *0401 females as compared to *0401 males and *0402 females. Interestingly CCL20 and CCL22 which are required for the generation of regulatory CD4 T cells and DCs, are reduced several fold in *0401 females as compared to *0401 males and *0402 females. These data suggest that events in gut may be involved in pathogenesis.

Key accomplishments as stated in SOW

Milestone 1-7 were achieved in last year progress report.

Milestone#8 Histopathology of in vivo studies and antibodies in mice treated with P. histicola have been completed.

Milestone #9 Data is being analyzed for publication.

Milestone# 10 Isolationof cells from intestine has been standardized.

Milestone #11 Immunomodulatory effect of bacterial treatment was studied by comparison of mRNA expression levels of various cytokines by rtPCR of cytokines in jejenum of treated and untreated mice.

Milestone #12 Publication of in vivo data is in progress.

Milestone #13 DR4.TLR4-/- have been generated and are being characterized.

Milestone #14 Modulation of cytokines by bacteria in spleen has been studied by using bioplex array system.

Publications and presentations

1.Taneja V. Gut and Autoimmunity. " Invited talk" 5th Federation of Immunological Societies Association. New Delhi, India, March 2012.

2. Taneja V. Collagen-induced arthritis, mouse model in humanized mice. " Invited Talk" Be the cure, Ist workshop on Standardizing procedure for experimental model, Stcokholm, Sweden, March 2012.

3. Luckey D, Behrens M, Karo M, Patel R, Murray J, Mangalam A and Taneja V. Microbial mucosal modulation of arthritis. "Microbiota and Mucosal Immunology Meeting: Interface in health and disease" San Francisco, April 14, 2011.

4. Gomez A, Yoeman C, Luckey D, Marietta EV, Miller ME, Murray JA, White BA and Taneja V. 2012. HLA-DR polymorphism, gut microbiome and sex may predict susceptibility or resistance to arthritis in humanized mice. PloS ONE 7(4):e36095. doi:10.1371 / journal .pone.0036095.

Reportable Outcome:

P. histicola generates immunosuppression in humanized mice and may be useful for clinical trials in RA patients.

Conclusions. Our in vitro and in vivo data showing suppression of antigen-specific immune response and arthritis in P. histicola treated mice suggests generation of peripheral tolerance via gut. Our studies show that treatment with P histicola suppresses arthritis via gut and generation of regulatory DCs and T regulatory cells. Our ongoing studies on the role of TLR4 will delineate if innate immunity is involved in antigen-specific tolerance in humanized model of arthritis.

References

1. Gregersen, P. K., J. Silver, and R. J. Winchester. 1987. The shared epitope hypothesis. An approach to understanding the molecular genetics of susceptibility to rheumatoid arthritis. *Arthritis and rheumatism* 30:1205-1213.
2. Taneja, V., N. K. Mehra, A. N. Chandershekaran, R. K. Ahuja, Y. N. Singh, and A. N. Malaviya. 1992. HLA-DR4-DQw8, but not DR4-DQw7 haplotypes occur in Indian patients with rheumatoid arthritis. *Rheumatology international* 11:251-255.
3. Vaahtovuo, J., E. Munukka, M. Korkeamaki, R. Luukkainen, and P. Toivanen. 2008. Fecal microbiota in early rheumatoid arthritis. *J Rheumatol* 35:1500-1505.
4. Taneja, V., M. Behrens, E. Basal, J. Sparks, M. M. Griffiths, H. Luthra, and C. S. David. 2008. Delineating the role of the HLA-DR4 "shared epitope" in susceptibility versus resistance to develop arthritis. *J Immunol* 181:2869-2877.
5. Taneja, V., M. Behrens, A. Mangalam, M. M. Griffiths, H. S. Luthra, and C. S. David. 2007. New humanized HLA-DR4-transgenic mice that mimic the sex bias of rheumatoid arthritis. *Arthritis and rheumatism* 56:69-78.
6. Taneja, V., and C. S. David. Role of HLA class II genes in susceptibility/resistance to inflammatory arthritis: studies with humanized mice. *Immunological reviews* 233:62-78.
7. Luckey D, Behrens M, Karo M, Patel R, Murray J, Mangalam A and Taneja V. Microbial mucosal modulation of arthritis. "**Microbiota and Mucosal Immunology Meeting:** Interface in health and disease" San Francisco, April 14, 2011.
8. Gomez A, Yoeman C, Luckey D, Marietta EV, Miller ME, Murray JA, White BA and Taneja V. 2012. HLA-DR polymorphism, gut microbiome and sex may predict susceptibility or resistance to arthritis in humanized mice. PloS ONE 7(4):e36095. doi:10.1371 / journal .pone.0036095

Microbial mucosal modulation of arthritis

David Luckey, Marshall Behrens, Melissa Karou, Robin Patel, Joseph Murray, Ashutosh Mangalam and Veena Taneja

Department of Immunology, Gastroenterology and Microbiology, Mayo Clinic Rochester, MN - 55901 USA.

Predisposition to rheumatoid arthritis (RA) is associated with the presence of genetic factors, HLA class II molecules, DR4 and DQ8, being the strongest. Recent reports that patients with RA have decreased fecal levels of certain commensal bacteria suggested that intestinal microbes might be critical in regulation of disease. We isolated *Prevotella histicola*, anaerobic commensal bacteria of Human gut, from bowel of a patient and have shown that it possesses anti-inflammatory activity. We propose that gut microbiota can influence peripheral immune response and may modulate arthritis in a murine model. We have established a murine model of rheumatoid arthritis using mice expressing RA-associated HLA genes, DRB1*0401 and DQ8. DR4 and DQ8 mice develop collagen-induced arthritis (CIA) following immunization with type II collagen (CII). We have used HLA-DR4/ DQ8 mice to test our hypothesis that treatment with commensal bacteria like *Prevotella histicola* can modulate CIA. In vitro data showed that treatment of mice with *P. histicola* in CII-immunized mice led to suppression of antigen-specific immune response and reduction in production of inflammatory cytokines suggesting P histicola has anti-inflammatory properties in this model. Treatment of CIA in transgenic mice in therapeutic protocol is ongoing. Our data suggests that *P histicola* induced immune responses in the gut causes systemic immune suppression and may be able to regulate autoimmunity.